Bactericide against *Streptococcus mutans* and *Streptococcus sobrinus*

Field of the Invention

The present invention relates to an enzyme having a bacteriolytic activity against *Streptococcus mutans* and *Streptococcus sobrinus,* and a means for protecting and treating tooth decay using the enzyme, and more particularly to toothpaste, gum and the like using the enzyme having a purpose of protection and treatment of tooth decay.

Prior Art

It has been elucidated based on numerous experimental studies using germfree rats and epidemiological studies that cariogenic bacteria inducing human tooth caries are *Streptococcus mutans* and *Streptococcus sobrinus* belonging to the group of streptococci (reference 3). During the study of bacteriolytic enzyme, which decomposes metabolically a big construct, peptidoglycan, contained in bacteria, the present inventors are interested in and are studying bacteriolytic enzyme produced by *Streptococcus mutans* (reference 4). Peptidoglycan is a construct involved in only eubacteria and archaebacteria among various living organisms, which has a mesh wire structure textured with sugar and peptide chains, and enwraps a bacterial cell. The structure of peptidoglycan is comparable to a bone structure maintaining a bacterial shape in order to hold the inner pressure with about 20 atms. Peptidoglycans have been considered as a target of antibacterial chemotherapeutic agents for a long time because of their specificities. A lot of antibiotic therapeutic agents, including β-lactam antibiotics such as penicillin G making a dent of antibiotics, are agents with their targets on biosynthesis of peptidoglycan systems. β-lactam medications have excellent selective toxicity because of lack of targets on animal cells and have been widely used as medical agents with minimal side effects.

On the other side, Hisae Baba et al. reported an enzyme, AL-7, with similar characteristics to the enzyme of the present invention produced by *S.mutans* (reference 5~7) and elucidated that the enzyme, AL-7, lyses selectively heated bacterial bodies of *Streptococcus sanguis* and *Streptococcus mutans.*

In addition to the above example, some examples concerning the enzymes produced by *S. mutans* have been reported (references 1~2 and others).

Reference 1: Japan Patent JP H10-136976

Reference 2: Japan Patent JP 2002-114709

Reference 3: Journal of Japanese Soc. for Bacteriology 51(4): 931-951, (1996)

Reference 4: ibid. 52(2): 461-473, (1997)

Reference 5: J. Oral Biol.,25:947-955, 1983

Reference 6: J. Oral Biol.,26:185-194, 1984

Reference 7: Kanagawa Odontology,24-2, 384-392, 1989

Problems to be solved by the Invention

Previously, it has been generally accepted as a concept of antibacterial chemotherapy that medical agents have targets common to various bacteria and that preferred action affects lethally to the targets. However, the action affects not only to bacteria targeted by chemotherapy but also to bacterial group forming normal bacterial flora, and induces replacement of bacteria. Furthermore, once bacteria acquire resistance to medical agents, rapid spreading of the resistance beyond a barrier of bacterial species will be recognized. Therefore, antibacterial chemotherapeutic agents, different from previous antibacterial agents, has been sought, which is effective to specific cariogenic bacteria.

Namely, the purpose of the present invention is to provide an enzyme attacking selectively cariogenic bacteria and a means for preventing and treating tooth decay using the enzyme.

Means to solve the Problems

Bacteriolytic enzyme is an enzyme essential for metabolyzing peptidoglycans during growth phase, wherein bacteria undergo mitosis and cell segregation. The present inventors discovered bacteriolytic enzyme Lyt100 produced by *Streptococcus mutans* during investigations, cloned the gene, constructed recombinants and examined the function of the enzyme. During the examination of the substrate specificity of the enzyme, the inventors discovered that the enzyme has a substrate specificity to lyse selectively *Streptococcus mutans* and *Streptococcus sobrinus*. The enzyme, which lyses selectively *Streptococcus mutans* and *Streptococcus sobrinus*, has advantages in lysing the cariogenic bacteria without affecting normal bacterial flora existing in mouth. Use

of the enzyme enables to remove selectively cariogenic bacteria or to decrease the number of cariogenic bacteria inside oral cavity, and may exert preventive effect against tooth caries.

Namely, the present invention is a bactericide against *Streptococcus mutans* and *Streptococcus sobrinus* comprising any one of the following proteins (1) to (3):

(1) a protein shown by the amino acid sequence of SEQ ID NO: 1 or a protein having the amino acid sequence derived therefrom in which one or more amino acids (for example, maximum 5%of total amino acids.) are deleted, substituted of added and having a lytic activity against *Streptococcus mutans* or *Streptococcus sobrinus*.

(2) a protein having a 100±10 kDa band of lysed bacteria in a zymography containing killed *Streptococcus mutans*.

(3) a protein obtained from cultured cells transformed by DNA comprising nucleotide sequence of SEQ ID NO: 2 or DNA encoding said protein (1)

Furthermore, the present invention is a preventive agent of tooth decay, a therapeutic agent of tooth decay, a toothpaste, an oral cavity cleaner or a preventive gum of tooth decay, containing the bactericide. Prescription of the above agents are according to conventional means of various fields.

Moreover, the present invention is a method for killing selectively *Streptococcus mutans* and *Streptococcus sobrinus* using any one of the following proteins (1) to (3):

(1) a protein shown by the amino acid sequence of SEQ ID NO: 1 or a protein having the amino acid sequence derived therefrom in which one or more amino acids are deleted, substituted of added and having a lytic activity against *Streptococcus mutans* or *Streptococcus sobrinus*.

(2) a protein having a 100±10 kDa band of lysed bacteria in a zymography containing killed *Streptococcus mutans*.

(3) a protein obtained from cultured cells transformed by DNA comprising nucleotide sequence of SEQ ID NO: 2 or DNA encoding said protein (1).

Brief Description of the Drawings

Figure 1 is the zymogram of the enzyme Lyt100 of the present invention

Figure 2 is the column chromatogram of the enzyme Lyt100 of the present invention using TSKgek Phenyl-5PW. Underline shows the positions with lytic activity.

Figure 3 is the electrophoresis profiles and the zymogram of the crude enzyme.

Figure 4 is the lytic activity of the enzyme Lyt100 of the present invention against killed bacteria. The ordinate and horizontal axes show turbidity (generally, absorbance at 660 nm) and time (min.), respectively.

Figure 5 is the lytic activity of the enzyme Lyt100 of the present invention against killed bacteria. The ordinate and horizontal axes show turbidity (generally, absorbance at 660 nm) and time (min.), respectively.

Figure 6 is the lytic activity of the enzyme Lyt100 of the present invention against killed bacteria. Relative turbidity at 180 min to that using S. mutans as a substrate is shown by %.

Figure 7 is the lytic activity of the enzyme Lyt100 of the present invention against vaiable bacteria. The ordinate and horizontal axes show turbidity (generally, absorbance at 660 nm) and time (min.), respectively.

Figure 8 is the lytic activity of the enzyme Lyt100 of the present invention against viable bacteria. Relative turbidity at 180 min to that using S. mutans as a substrate is shown by %.

Figure 9 is the bactericidal activity of the enzyme Lyt100 of the present invention against viable bacteria. The ordinate and horizontal axis show number of colonies (number of viable bacteria) and time (min.), respectively.

Figure 10 is the bactericidal activity of the enzyme Lyt100 of the present invention against viable bacteria. The ordinate and horizontal axes show number of colonies (number of viable bacteria) and time (min.), respectively.

Detailed Description of the Invention

The enzyme Lyt100 of the present invention is different from the enzyme AL-7 produced by *Streptococcus mutans*. (reference 5), although AL-7 has a similar characteristics to Lyt100 of the present invention. First of all, AL-7 is an extracellular enzyme, while Lyt100 is an intracellular enzyme. 20 mg AL-7 enzyme sample shows maximum 17% and 20.6% lytic activity against heat killed *Streptococcus mutans* and *Streptococcus obrinus*, respectively; and shows 6% and 8.3% lysing activity against cell wall of *Streptococcus mutans* and *Streptococcus sobrinus*, respectively. On the other hand, by using the similar assay system, 3 μg Lyt100 enzyme shows maximum 23% and 33.6% lytic activity against heat killed

Streptococcus mutans and *Streptococcus sobrinus*, respectively; and shows 96.7% and 96.7% lysing activity against cell wall of *Streptococcus mutans* and *Streptococcus obrinus*, respectively. Lyt100 has a stronger lysing activity agaist cell wall than AL-7. Whereas, for viable cells, 20 mg AL-7 enzyme shows maximum 3.2% and 3.3% lytic activity against *Streptococcus mutans* and *Streptococcus sanguis*, respectively, i.e. AL-7 enzyme has almost no lytic activity against viable cells and has no species specificity. On the contrary, 10 μg Lyt100 enzyme shows 44% and 56% lytic activity against *Streptococcus mutans* and *Streptococcus sobrinus*, respectively, and 0 % lytic activity against *Streptococcus sanguis*, *Streptococcus salivarius* and *Streptococcus mitis*, i.e. Lyt100 enzyme has a strong lytic activity with species specificity against *Streptococcus mutans* and *Streptococcus sobrinus*.

Lyt100 enzyme of the present invention is an enzyme produced in a pathogenic bacteria (*Streptococcus mutans*) and lyses and kills the same pathogenic bacteria themselves. Since the enzyme has strong species specificity and does not affect to other bacterial flora, it can be applied for treatment and protection of decayed tooth.

The following examples illustrate the present invention more clearly, but it is not intended to limit the scope of the present invention.

Example 1

(1) Preparation of crude enzyme

After *Streptococcus mutans* strain MT703R (hereinafter, *S. mutans*) was cultured in 600 ml brain-heart-infusion medium at 37°C overnight, cells were centrifuged at 8000 x g for 20 min and a pellet (about 1.2 g) was obtained. The pellet was added 2 ml of 8 M urea, was suspended and was left to stand at room temperature for 30 min. The suspension was centrifuged at 15,000 x g for 15 min and the supernatant was obtained. The supernatant was concentrated in a membrane ultrafilter (Amicon). The final concentration was adjusted to 1 mg / ml and it was used as crude enzyme.

(2) Discovery of lytic enzyme Lyt100

The crude enzyme was applied to a zymography. A zymography is a method of applying SDS polyacrylamide gel electrophoresis for assaying a lytic enzyme activity. Firstly, killed cells (1 mg / ml) of *S. mutans* were added to polyacrylamide gel at the time of gel

polymerisation. Then, after usual electrophoresis, the gel was washed with water, was incubated in 0.1 M phosphate buffer (pH 7.0) to recover the lytic enzyme activity inside the gel. The recovered lytic enzyme lyses the killed cells near the protein band and leads to be detected as a transparent band with a background of white turbid gel. The obtained gel is referred to as zymogram.

The killed cells of *S. mutans* was used after treatment of cells with 100°C hot water / 4% SDS for 30 to 60 min and subsequently after washing with enough volume of PBS for ten times.

As shown in Figure 1, two lytic bands were observed in the region of high molecular weight. After the SDS gel electrophoresis of the crude enzyme, the protein in the gel was stained with Coomassie brilliant blue and the protein bands corresponding to the lytic band were checked by comparing to the zymogram. The two protein bands contained in the gels (corresponding to two lytic activities) were cut out, were transferred to a Nylon (R) membrane and were applied to gas phase amino acids sequence analyzer (Model 49X Procise). Based on the obtained amino acids sequence (SEQ ID NO: 1), DNA fragment comprising the nucleotide sequence (SEQ ID NO: 2) corresponding to the two amino acids sequence was found by using TIGR unfinished *Streptococcus mutans* UAB159 DNA sequence database.

The obtained two DNA fragments encode the same protein with different sizes. The parent protein was secreted on a cell surface after biosynthesis and was partially digested by another proteinase. Namely, it was found that Lyt100 had signal sequence with 24 amino acids and the size of the mature form was 104.424kDa. Partial digestion of the mature form protein removed amino-terminal 182 amino acids and resulted in 89.680 kDa.

Primers (SEQ ID NOs: 3, 4) were prepared based on DNA encoding the full-length protein and DNA encoding the mature form enzyme protein was amplified using *S. mutans* C67-1 chromosome as a template. The DNA was inserted into an expression vector pQE30 and was transfected into *E. coli* M-15. One of the obtained transfomants was named as GY122.

(3) Purification of recombinant lytic enzyme Lyt100

E. coli GY strain 122 was cultured in 500 ml of LB liquid medium (for about 4 hrs), was added final 1 mM isopropyl-D-thiogalactopyranocide when absorbance at 660 nm was 0.5. After further 3 hrs culture, the culture medium was centrifuged. After 30 min centrifugation

at 8,300 g, the pellet was suspended in phosphate buffered-saline (PBS) (10 ml PBS for 1 g cell pellet), then the procedures of suspending and centrifugation were repeated for two times. The pellet finally obtained was suspended in phosphate buffered-saline (PBS) (10 ml PBS for 1 g cell pellet), was sonicated in ice-cold water (Tomy Seiko level 4, 50% interval, 20 min), and was centrifuged. The obtained pellet was suspended in PBS containing 0.2% Triton X-100 (10 ml PBS for 1 g pellet) and was left to stand at room temperature for 30 min. The above procedure was repeated again and the obtained pellet was dissolved in 8 to 10 volumes of 8 M urea, 0.1 M Na_2PO_4, 0.01 M Tris-HCl (pH 8.0). Ni-NTA resin beads (1 ml) was added to the obtained solution, was washed with 8 M urea, 0.1 M Na_2PO_4, 0.01 M Tris-HCl (pH 6.3) and was eluted by 8 M urea, 0.1 M Na_2PO_4, 0.01 M Tris-HCl (pH 5.4). Each fraction was 500 µl and the 15th to 20th fractions were collected. Each fraction was assayed for lytic activity, active fractions were collected and were dialyzed against 0.1 M phosphate buffer containing 1 M NaCl, 1 M urea at 4°C for overnight. The dialysate was charged on a TSKgel Pheny-5PW (75 mm x 7.5 mm, lot 5PHR0050) column of high performance liquid chromatography, which had been equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 1 M NaCl and 1 M urea (A buffer). After washing with enough volume of the buffer, the A buffer was linearly changed to B buffer (0.1 M phosphate buffer, pH 7.0, containing 1 M urea) with a flow rate of 0.5 ml / min in 30 min to elute the active fraction. As shown in Figure 2, the active fractions were eluted at the positions shown by a solid line.

Figure 3 shows SDS-gelelectrophoresis profiles for the sample before purification and after purification. The result that Lyt100 was electroporesed at the position of about 100 kDa (100±10 kDa) shows that the desired protein was purified.

Example 2

(4) Measurement of lytic activities using killed cells

As oral streptococci, the following 5 strains were used: *S. mutans* C67-1, *S. sobrinus* OMZ176a, *S. mitis* ATCC9811, *S. sanguis* ATCC10436, and *S. salivarius* ATCC9222.

Heat killed bacterial cells in boiled water containing 4% SDS were washed with enough amount of water and were suspended in turbidity buffer (0.1 M phosphate buffer, 0.1 M NaCl, 1 mM Ca, pH 6.8) by adjusting absorbance to 0.3 at 660 nm. The purified Lyt100 was added to 2 ml cell suspension and the time course of the absorbance change was recorded.

The lytic activity against killed cells is shown in Figs 4 to 6. Lyt100 has strong lytic

activity against *S. mutans* C67-1 and *S. sobrinus* OMZ176a, especially the activity against *S. sobrinus* OMZ176a was two times of that against *S. mutans*.

Example 3

(5) Measurement of lytic and bactericidal activities using viable bacteria.

As oral streptococci, the following 5 strains were used: *S. mutans* C67-1, *S. sobrinus* OMZ176a, *S. mitis* ATCC9811, *S. sanguis* ATCC10436, and *S. salivarius* ATCC9222.

Cultured various strains of bacteria were suspended in turbidity buffer. In order to disperse the linkage of bacteria, *S. mutans* were sonicated at level 4 for 10 sec and other streptococci were sonicated at level 4 for 5 sec. Then, they were suspended in the buffer adjusting absorbance to 0.5 at 660 nm. Purified Lyt100 was added to 2 ml suspension and the time course of the change of absorbance was recorded. At the same time, the aliquots of the samples were diluted to 10^4- to 10^5-fold and were seeded on brain-heart-infusion agar media for *S. mutans* C67-1, *S. sobrinus* OMZ176a, *S. salivarius* ATCC9222 and on MS agar media for *S. mitis* ATCC9811, *S. sanguis* ATCC10436. Then viable number of colonies was counted.

Figures 7 and 8 show lytic activity against viable bacteria. Generally, viable bacteria are less sensitive against enzyme than killed bacteria. Lyt100 was used at 3 µg / 2 ml in the lytic assay against killed bacteria, but at 10 µg / 2 ml in that against viable bacteria. Even in the latter case, Lyt100 has strong lytic activity against *S. mutans* C67-1and *S. sobrinus* OMZ176a.

Figures 9 and 10 shows the bactericidal activity against viable bacteria. Colony forming unit was calculated for viable bacterial suspension treated with Lyt100 and the results were paralleled to that of turbidity decrease. It was found that Lyt100 had selective bactericidal effect against *S. mutans* C67-1and *S. sobrinus* OMZ176a.